REVIEW
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The biology of E colicins: paradigms and paradoxes

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Norwich NR4 7TJ, UK**Keywords:** E colicins, receptor-binding, colicin translocation, protein–protein interaction, evolution

Overview

Colicins are plasmid-encoded antibacterial proteins that are classified into groups on the basis of the cell surface receptor to which they bind. The E colicins, for example, all bind to the product of the chromosomal *btuB* gene, which is an essential component of the high-affinity transport system for vitamin B₁₂ (cobalamin) in *Escherichia coli* (Di Masi *et al.*, 1973). Each E colicin plasmid encodes the production of a specific immunity protein which protects the producing cell against the cytotoxic activity of its colicin, located at the C-terminus of the protein, by binding to this domain (Jakes *et al.*, 1974; Sidikaro & Nomura, 1974; Wallis *et al.*, 1992a). Based on immunity tests (Watson *et al.*, 1981; Cooper & James, 1984) the E group colicins have been subdivided into nine types (ColE1–E9), and these fall into one of three cytotoxic classes: membrane-depolarizing (or pore-forming) agents such as ColE1 (Cramer *et al.*, 1990), DNases such as colicins E2, E7, E8 and E9 (Lau *et al.*, 1992), and RNases such as colicins E3, E5 and E6 (Masaki *et al.*, 1992). *E. coli* K12 strains carrying an E colicin plasmid are sensitive to the DNA-damaging agent mitomycin C, in contrast to plasmid-free isogenic strains (Shafferman *et al.*, 1979). This is due to the induction of a DNA-damage-inducible promoter (SOS promoter), located proximal to the E colicin structural gene, which results in enhanced expression of both the colicin gene and of another plasmid-encoded gene (*lys* or bacteriocin-release protein, BRP). The BRP is involved in release of the colicin–immunity protein complex from the producing cell and causes partial cell lysis upon strong induction of the SOS promoter (Pugsley & Schwartz, 1983; Suit *et al.*, 1983).

Scope of the review

In this review we will discuss the progress in our understanding of the mode of action and inhibition of the nuclease-type E colicins, which have been much less well studied and reviewed compared to the pore-forming

colicins such as colicins E1 and A (see for example, Cramer *et al.*, 1990; Lazdunski, 1995). Nuclease-type E colicins are multidomain proteins that exhibit a number of fundamental biological properties (Fig. 1). The specific

Fig. 1. Schematic diagram illustrating the biological problems addressed by studying the mechanism of action and immunity of nuclease-type E colicins. The colicin E9 protein is subdivided into the 15 kDa DNase domain, to which Im9 binds, and the 45 kDa receptor-binding and translocation domain. The colicin E9–Im9 complex is released from the producing cell and then binds to sensitive *E. coli* cells via the BtuB receptor. The fate of Im9 and the exact nature of the (presumed) fragment which initiates the cytotoxic activity is unknown.

interaction between the C-terminal domain of the colicin and its cognate immunity protein prevents the cytotoxic domain meeting its cellular target and is a model system for studying protein-protein interactions. The extracellular release of the colicin-immunity protein complex from the producing *E. coli* cell represents an unusual protein secretion system in which the *lys* gene product, rather than the secreted proteins, contains a signal sequence. This system has been used to facilitate the extracellular release and subsequent purification of a number of recombinant-DNA-encoded proteins (van der Wal *et al.*, 1995). The mechanism by which colicins kill cells is a rare example of a polypeptide uptake system into bacterial cells. Entry goes by two discernible steps: first binding to the receptor, followed by translocation of the cytotoxic domain across the two bacterial membranes to the cytoplasm of the sensitive *E. coli* cell. A detailed understanding of these processes, which are also used for the uptake of essential metabolites, may have applications in the development of new antibiotics. The existence of a family of DNase-type and of RNase-type E colicins has facilitated the study of protein-protein interactions between the colicins and their cognate immunity proteins, but also raises interesting questions about the evolution of E colicins and their plasmids.

Protein-protein interactions between E colicins and immunity proteins

Immunity of colicinogenic cells towards the action of the colicin they produce was first noted by Fredericq in 1957, and since that time much progress has been made in studying the biochemical basis of this activity. In the early 1970s it was demonstrated that the activity (for the RNase colicin ColE3) could be detected in cytoplasmic extracts (Jakes *et al.*, 1974). There then followed a series of investigations from several laboratories in which the immunity proteins for RNase- and DNase-type colicins were characterized (Chak & James, 1984; Lau *et al.*, 1984a, b), their association to their respective colicin studied, and the domain structure of the colicins themselves elucidated (Ohno *et al.*, 1977; Yamamoto *et al.*, 1978). Over the last few years, the genes for many of the colicins and their immunity proteins have been cloned and sequenced and, more recently, investigations have begun, using recombinant DNA techniques coupled with biophysical analysis, into the specificity determinants of both RNase- and DNase-type colicins and their immunity proteins.

RNase colicins

Masaki and co-workers have focused on the RNase colicins E3 and E6 and the functionally analogous bacteriocin from *Klebsiella pneumoniae*, cloacin DF13. Their work has shown that the determinants for specificity of the colicin-immunity protein interaction are likely to reside in a limited number of amino acid residues, eight in the nuclease domains and up to nine in the corresponding immunity proteins (Akutso *et al.*, 1989). In both cases, these determinants are located in the N-terminal region of

the protein. Further work on the RNase immunity proteins using homologous recombination showed that a single residue at position 48, a tryptophan in Im6 but a cysteine residue in Im3, was critical for defining specificity (Masaki *et al.*, 1991). Other residues, such as Glu-19 are also thought to be involved in the interaction, but seem to have a more peripheral role in defining specificity. The Japanese group also went on to determine the three dimensional structure of Im3 by high-field NMR (Yajima *et al.*, 1992). The protein contains 84 amino acids and is folded into a four-stranded, antiparallel β -sheet connected by loops and a single short α -helix. This is in marked contrast to the recently determined structure of a DNase-specific immunity protein, Im9 (Osborne *et al.*, 1994, 1996, and unpublished results), which contains four α -helices (see below). The specificity determining residues of Im3 at positions 19 and 47 are exposed and line one face of the β -sheet (Yajima *et al.*, 1992). No structures are yet available for any of the RNase domains, although crystals of both Im3 (Shoham *et al.*, 1984) and the colicin E3-Im3 complex have been reported (Frolow & Shoham, 1990).

DNase colicins

Our work on colicin immunity-specificity has focused on the interaction between DNase colicins and their immunity proteins. At the time of our original experiments on specificity, no structural information was available, so our studies were guided by sequence alignments, in combination with site-directed mutagenesis and/or gene fusion experiments. The nucleotide sequence of the E2, E7, E8 and E9 *imm* genes are known, as are the complete nucleotide sequences of colicins E2 (*ceaB*), E7 (*ceaG*) and E9 (*ceaI*), together with the sequence of the 3' end of the colicin E8 structural gene (*ceaH*). The degree of homology between the DNase domains of colicins E2, E7, E8 and E9 is > 80%. In a study of the specificity determinants of colicins E9 and E8 for their cognate immunity proteins we used site-directed mutagenesis, in conjunction with sequence alignments between ColE8 and E9, to analyse the specificity differences between the two colicin proteins (Curtis & James, 1991). Six ColE9 residues in the C-terminal DNase domain were identified as possible specificity determinants and changing all six to the corresponding amino acids of ColE8 changed the phenotype of the 'mutant' protein to one which exhibited ColE8 activity, but which still possessed significant ColE9 activity.

The amino acid sequences for the four immunity proteins, Im2, Im7, Im8 and Im9, are 48% identical and 58% homologous (Fig. 2), and show no apparent homology to the RNase-type immunity proteins. The degree of specificity within the DNase immunity proteins is exemplified by the fact that, at normal levels of expression, the E9 immunity protein (Im9) will only protect *E. coli* cells against externally added colicin E9, and not against colicins E2, E7 or E8 (Cooper & James, 1984). This is similar to the situation in the RNase-type colicins E3 and E6 which share a high degree of sequence identity, both in their nuclease domains (> 80% identical) and their immunity proteins (> 70% identical), but show no cross-reactivity

Fig. 2. Sequence alignment for the E colicin endonuclease-immunity protein family. Overlaid on the sequence alignment is the secondary structure distribution of Im9, the structure of which has been solved by NMR (Osborne *et al.*, 1994, 1996). The sequence of Im8 is one residue shorter than Im9 while that of Im7 is one residue longer. The padding character/insertion in the alignment to compensate for these differences has been made in the loop connecting helices I and II, which therefore makes the assumption that the length of these two helices will be the same in all the immunity proteins. The figure also shows the specificity-determining region (SDR) of the immunity proteins, identified by homologous recombination experiments (Wallis *et al.*, 1992b). Asterisks indicate amino acid residues of Im9 that have been substituted for those of Im8; the substitution at residue 34 substantially affects immunity protein specificity while that at 37 is less effective (Wallis *et al.*, 1992b). The solid bars above the secondary structure map show the amide regions of Im9 that are perturbed on binding to the E9 DNase as detected by ¹H-¹⁵N heteronuclear single-quantum coherence (HSQC) experiments (M. J. Osborne and others, unpublished results); thick bars represent chemical shift changes of between 1-3 p.p.m. in the ¹⁵N dimension and/or 0.2-0.6 p.p.m. in the ¹H dimension, and thin bars represent chemical shift changes of 0.2-1 p.p.m. in the ¹⁵N dimension and 0.04-0.2 p.p.m. in the ¹H dimension.

under normal levels of expression (reviewed in Lau *et al.*, 1992). The specificity-determining region (SDR) in the Im8 and Im9 immunity proteins (Fig. 2) was found by homologous recombination experiments to lie between residues 16 and 43 (Wallis *et al.*, 1992b). Subsequent single and multiple residue swap mutations identified two Im9 residues which, when changed to their Im8 equivalents, resulted in a significant level of immunity to colicin E8 without any loss in immunity to colicin E9 (Wallis *et al.*, 1992b). The amino acid substitution in Im9 that caused the most significant change in specificity was Val34Asp; in plate assays the resulting mutant retained ColE9-specificity but also showed significant cross-reactivity towards ColE8. The other mutation site Val37Glu showed much less cross-reactivity. Further analysis of this mutant, coupled with the structural information now available on the Im9 protein (Osborne *et al.*, 1994, 1996, and unpublished results), suggests that the original sequence alignment, which identified a glutamic acid in the 37 position of Im8 corresponding to the valine of Im9, is probably incorrect. Position 37 is a buried residue in the structure of Im9. The equivalent residue in all the other DNase-specific immunity proteins is also a bulky, hydrophobic amino acid. Characterization of the Val37Glu Im9 mutant has shown that it is highly destabilized, which is consistent with it being buried in the folded protein. This is in marked contrast to the Val34Asp Im9 protein which shows the same stability as wild-type Im9 (unpublished observations). While it is unclear why the Val37Glu Im9 mutant shows cross-reactivity towards the ColE8 toxin, one reasonable explanation would appear to be that it is due to the introduction of negative charge in a region of Im9 which, in the folded protein structure, is close to residue 34, a *bona fide* specificity site.

Structural and biophysical analysis of DNase-immunity protein complexes has been aided by the *E. coli* over-expression systems developed, first for the 9.5 kDa Im9 protein (Wallis *et al.*, 1992a), and subsequently for the 15 kDa DNase domain of ColE9 (the E9 DNase) (Wallis *et al.*, 1994). Similar strategies have now been used to overexpress the remaining DNase domains and immunity proteins of this family (unpublished observations). The affinity for a colicin-DNase-immunity-protein complex has been defined for the E9 system using a combination of stopped-flow fluorescence (to determine k_{on}) and radioactive exchange kinetics (to determine k_{off}) (Wallis *et al.*, 1995a). The colicin E9-Im9 complex is extremely stable ($k_{off}/k_{on} = K_d = 9.3 \times 10^{-17}$ M), and this is very similar to the stability of the isolated DNase-domain-Im9 complex ($K_d = 7.2 \times 10^{-17}$ M). These affinities are some of the highest ever measured for a protein-protein interaction. The work also showed that the association of the nuclease with the Im9 protein is essentially diffusion-controlled, involves electrostatic steering and follows a two-step mechanism in which the proteins form an initial encounter complex prior to undergoing a conformational change to yield the final stable complex (Wallis *et al.*, 1995a).

One of the great surprises of biophysical studies has been that all the DNase-specific immunity proteins can bind to the DNase domain of colicin E9 and inhibit its activity (Wallis *et al.*, 1995b). Moreover, the association kinetics for the non-cognate Im proteins, Im2 and Im8, were almost identical to those of the cognate protein Im9. The question therefore arises as to how specificity is defined in this system. The answer appears to be that specificity is controlled through the dissociation rate constant for these protein complexes which are $> 10^6$ -fold faster for the non-cognate Im proteins. This results in affinities for the

Fig. 3. Structural models of Im9. Ribbon diagram of the Im9 structure determined by NMR. The picture was generated using Molscript (Kraulis, 1991).

non-cognate complexes that are significantly lower than the cognate complex; thus the K_d values for the non-cognate Im proteins in complex with the E9 DNase are, Im2 10^{-8} M, Im8 10^{-6} M and Im7 10^{-4} M (Wallis *et al.*, 1995b). This 10^{12} -fold range in dissociation constants in a family of protein complexes is highly unusual and promises to throw new light on the controversial subject of specificity in protein-protein interactions. Recognizing that in fact all the Im protein family can bind to a single DNase colicin raises the issue of why cross-reactivity between colicin-producing cells is not readily observed. The answer seems to be simply one of expression levels. At the normal levels of expression of immunity genes in naturally producing bacteria, no significant cross-reactivity between the different DNase colicins is observed. However, overexpressing each of the non-cognate Im genes in bacteria resulted in significant levels of cross-reactivity towards the ColE9 toxin and the order of *in vivo* cross-reactivity (Im9 > Im2 > Im8 > Im7) mirrors exactly the measured *in vitro* affinities (Wallis *et al.*, 1995b).

NMR studies of Im9 have allowed its structure to be determined and has shed some light on the question of how it interacts with the colicin E9 DNase (Osborne *et al.*, 1994, 1996, and unpublished results). The Im9 fold is a novel and can be considered as a distorted anti-parallel four-helix bundle, in which the short helix III is terminated close to its N-terminal end by a proline at its C-terminus, Pro56 (Fig. 3a). The SDR between residues 16 and 43 forms the C-terminal end of helix I, which runs from residue 12 to 23, a linking loop between residues 24 and 29, and helix II, which runs from residues 30 to 44.

The loop is not well-defined in the structure suggesting that it is disordered in solution. Helix II is considered to be the main specificity-determining helix, partly because it carries Val34 and Val37 referred to above as specificity determinants. The side chain of Val34 is almost completely exposed to solvent whilst little of the Val37 side chain is exposed. Interestingly, the exposed region of Helix II abuts a region of the surface where invariant residues forming Helix III are highly exposed to solvent. The spatial relationship between Helix II and the invariant residues forming helix III, particularly tyrosines 54 and 55, is intriguing and, as further NMR experiments showed, provided the first clue as to how Im9 may interact with the DNase. By isotopically labelling Im9 with ^{15}N , but leaving the DNase unlabelled so that it contained > 99% ^{14}N , and then using ^{15}N NMR we were able to look at the bound Im9 and compare it with the free Im9. From the NMR parameters obtained in this experiment it was not possible to determine the structure of the bound Im9, but we were able to map the surface region of Im9 which interacts with the DNase (Osborne *et al.*, 1996, and unpublished results). This region includes Helix III as well as Helix II. This NMR experiment does not show which of the residues are particularly important for determining the high binding affinity of the Im9-E9 DNase complex but it does point to the possibilities. Pinpointing the key residues will require site-directed mutagenesis which, guided by the structural information, is currently underway. One of the features of the proposed binding surface that ties in with the kinetic studies referred to above is the distribution of charged residues. The binding surface is heavily negatively charged, exactly what would be anticipated for a protein interacting with a positively charged partner, as the DNase domain is, in an ionic-strength-dependent reaction. Intriguingly, the invariant Asp51 is close to Helix II and it will be interesting to see if this is important for influencing K_d .

Extracellular release of E colicins

Colicins are not the only class of proteins that are released into the growth medium by Gram-negative bacteria. However, they constitute a novel group which is different from other secreted toxins and enzymes (Cavard & Oudega, 1992). The characteristics of colicins that relate to their extracellular release are: (a) they do not contain either an N-terminal or an internal signal sequence; (b) their release relies on the expression of only one gene which encodes a bacteriocin-release protein (BRP), also called a lysis or Kil protein; (c) they are released from the host cell some time after synthesis; and (d) their extracellular release is not specific; they appear to be released with a subset of cytoplasmic and periplasmic proteins during 'quasi-lysis'.

The gene encoding the BRP is located downstream of the colicin and immunity genes as part of an operon. The organization of the three genes in the operon differs between the pore-forming colicins, such as colicin A and E1, and the enzymic colicins E2-E9 (Cavard & Oudega, 1992). In the former, the BRP gene is separated from the 3' end of the colicin structural gene by a region of about

550 bp which contains the gene encoding the immunity protein on the opposite DNA strand. A transcription terminator is located between the colicin structural gene and the immunity gene. In the enzymic colicins, the BRP gene is located proximal to the immunity gene with a transcription terminator located between the two genes. Transcription of the BRP gene in each case is regulated by the SOS promoter located upstream of the colicin structural gene. Most experimental work has concentrated on the BRPs from cloacin DF13, colicin E1, or colicin A, but the sequence similarity between the BRPs (Cavard & Oudega, 1992) makes it reasonable to assume that the BRPs of the nuclease-type E colicins will exhibit similar properties.

The BRPs are synthesized as precursors with a signal sequence. They mediate three functions: (a) non-specific colicin release; (b) cell lysis; and (c) killing of the producing cells. They are lipoproteins which are lipidated and processed in several steps (Wu & Tokunaga, 1986). The precursor form is acylated on the cysteine residue before being processed by signal peptidase II. After removal of the signal sequence the amino group of the acylated N-terminal cysteine is modified by a fatty acid residue. The maturation and processing of BRPs occurs slowly such that every intermediate form can be observed, and both the mature form and the signal sequence accumulate in *E. coli* cells after processing (Oudega *et al.*, 1984; Cavard *et al.*, 1987; Pugsley & Cole, 1987). The addition of globomycin to *E. coli* cells stops processing of the BRP and inhibits colicin release. Site-directed mutagenesis studies have demonstrated that acylation is essential for BRP function (Pugsley & Cole, 1987; Cavard *et al.*, 1987).

It has been proposed that the BRPs activate phospholipase A in the outer membrane of *E. coli* cells, which alters the permeability of the outer membrane and facilitates colicin release from the periplasm and cell lysis (Pugsley & Schwartz, 1984). This model is supported by the observation that *pldA* (phospholipase A) mutants do not release colicin or show cell lysis on induction of a colicin operon. However, the observation that the colicin A BRP is significantly degraded by the DegP protease in these mutants (Cavard *et al.*, 1989) may mean that the lack of colicin release and cell lysis is an artefact due to their inability to reach a concentration of the BRP sufficient to trigger secretion and cell lysis. Similarly, the colicin A BRP has been shown to have a phospholipase-A-independent effect upon the integrity of the *E. coli* outer membrane (Howard *et al.*, 1991). Other theories have been proposed that seek to explain the role of BRP in colicin release which involve either the formation of pores in the cytoplasmic and outer membranes (de Graaf & Oudega, 1986), or the fusion of the two membranes induced by vesicles formed from BRP molecules (Cavard *et al.*, 1985). It is thus apparent that considerable research needs to be carried out before we fully understand the role of BRP in colicin release. However, it is encouraging that the BRP system is being developed for the eventual purification of a number of recombinant-DNA-encoded proteins (Lloubès *et al.*, 1993; van der Wal *et al.*, 1995).

Engineering of the BRP by replacing its stable signal sequence with the signal sequence from the murein lipoprotein, which has a much shorter half-life in *E. coli*, improves expression and BRP-mediated release of the target protein (van der Wal, 1995). Mutagenesis of the Lpp-BRP offers the possibility of abolishing the quasi-lysis, whilst still releasing periplasmic proteins, thus increasing the efficiency of the process.

Binding of E colicins to the BtuB receptor

The BtuB protein is a minor component of the *E. coli* outer membrane, being present in about 200 copies per cell. The mature protein, after removal of the 20 amino acid signal sequence which is cleaved during secretion across the cytoplasmic membrane, consists of 594 amino acid residues and has a molecular mass of 66 kDa (Heller & Kadner, 1985). Mutations in the *btuB* gene reduce the transport of vitamin B₁₂ dramatically, and maximal growth of these mutants requires supplementation of the growth medium with 1–5 µM vitamin B₁₂ (Bassford & Kadner, 1977). As well as being the receptor for the E colicins and colicin A, BtuB also acts as the receptor for adsorption of bacteriophage BF23, which is closely related to phage T5. It has been shown that while all BtuB molecules in the outer membrane are able to transport vitamin B₁₂ and facilitate the lethal effects of phage BF23, only a proportion act as colicin receptors. It has been suggested that only newly synthesized BtuB molecules can mediate colicin action, possibly whilst they are associated with areas of contact between the inner and outer membranes (Luria & Suit, 1987).

In a study of nonsense suppression of chromosomal *btuB* mutants of *E. coli*, two phenotypic classes of mutant were observed (Hufton *et al.*, 1995). The first were defective in all BtuB functions (pleiotropic mutants), whilst the second category, which included Q62K, Q150K and Q299K, were resistant to phage BF23 but were similar to wild-type cells with respect to E colicin sensitivity and vitamin B₁₂ uptake. A two-dimensional folding model of the BtuB protein has been proposed which predicts 24 membrane-spanning β -strands (Hufton *et al.*, 1995). This model predicts that the three non-pleiotropic *btuB* mutants described above are located external to the outer membrane in loops exposed on the cell surface, in a position where they could be involved in ligand binding to BtuB. An alternative folding model has been proposed which predicts only 16 membrane-spanning β -strands (Bradbeer, 1991). Although it will be a major task, the determination of the three-dimensional structure of BtuB will be invaluable in our understanding of the function of this receptor in the binding of the three different ligands.

Fusions of alkaline phosphatase (encoded by *phoA*) to BtuB have been isolated throughout the BtuB polypeptide from amino acid 22 to 500 (Kadner *et al.*, 1992). All BtuB-PhoA fusions containing 22–327 amino acid residues of BtuB appeared to be located in the periplasmic space, whilst the BtuB-PhoA fusions containing more than 399 amino acids of BtuB appeared to be associated with the outer membrane. This suggests that amino acids

Fig. 4. Receptor-binding and translocation of group A colicins. The figure illustrates the receptor-binding and translocation pathways of group A colicins, two of which (colicin E1 and A) form pores in the cytoplasmic membrane, whilst the enzymic colicins, E2-E9, are translocated to the cytoplasm (see the text for a full discussion). For comparison, the figure also includes two group B colicins (B and D), which bind to the FepA receptor and also cause pore-formation in the cytoplasmic membrane, along with vitamin B₁₂ and Fe-DBS, all of which are translocated via the TonB-dependent uptake system. The final stages of the uptake of vitamin B₁₂ and Fe-DBS, at the cytoplasmic membrane, involves a number of gene products.

between 327 and 399 are required for association of BtuB with the outer membrane. Recently, a series of mutant forms of BtuB were constructed, which resulted either from the insertion of dipeptide sequences or duplications, or from in-frame deletions (Lathrop *et al.*, 1995). Even long duplications of as many as 340 repeated N-terminal residues retained function, suggesting considerable plasticity in the sequence requirements for membrane-insertion of BtuB.

Addition of vitamin B₁₂ to *E. coli* cells before treatment with either colicin A or E protects them from killing. Cells were also rescued from killing shortly after colicin treatment by the addition of vitamin B₁₂ (Cavard, 1994). Similar results were previously obtained by the addition of trypsin, or SDS, but unlike vitamin B₁₂, these reagents were able to rescue cells for a significantly longer period of time after colicin treatment. Vitamin B₁₂ rescue of colicin-treated cells may only be possible before irreversible binding of the colicin to BtuB, whereas trypsin and SDS can rescue cells at a later stage in the translocation process (see below).

Translocation of E colicins

Colicins bind to specific receptors in the outer membrane from where they are translocated across the outer

membrane. Translocation is mediated by the Ton or the Tol system. The group A colicins (A, E1-E9, K, L, N and S4) use the Tol system and require porins such as OmpF, together with the TolA, TolB, TolQ and TolR proteins (and TolC in the case of colicin E1) (Bénédetti *et al.*, 1989; Lazdunski, 1995); whilst the group B colicins (B, D, G, H, Ia, Ib, M, Q and V) use the Ton system and require specific receptors and the TonB, ExbB and ExbD proteins (Braun, 1995). Considerable progress has been made in understanding the interaction between a colicin molecule and components of the Tol-dependent or the Ton-dependent translocation pathways (Fig. 4).

The Tol-dependent translocation pathway is used both for the uptake of group A colicins and for the DNA of filamentous phages. Four gene products are involved, *tolQ*, *tolR*, *tolA* and *tolB*, which are clustered at 17 min on the *E. coli* chromosomal map (Webster, 1991). The TolA, TolB, TolQ and TolR proteins are required to translocate colicins A, E2-E9, K and N, whilst only the TolC, TolA and TolQ proteins are required to translocate colicin E1. The nucleotide sequence of the *tol* genes has been determined and the Tol proteins have been localized in the *E. coli* cell envelope (Fig. 4). The TolQ, TolR and TolA proteins are associated with the cytoplasmic membrane. TolQ contains three membrane-spanning segments (Kampfenkel & Braun, 1993). TolA and TolR are

anchored to the cytoplasmic membrane by their N-terminal domains, whilst TolB is principally located in the periplasm, but is associated with the outer membrane (Isnard *et al.*, 1994), through an interaction with the peptidoglycan-associated protein (Pal) (Bouveret *et al.*, 1995). TolC is an outer-membrane protein which is able to form pores *in vitro* (Benz *et al.*, 1993) and is also involved in the secretion of α -haemolysin (Wandersman & Delepeleire, 1990) and colicin V (Gilson *et al.*, 1990).

The OmpF porin is one of the major outer-membrane proteins of *E. coli* and forms three large water-filled channels per trimer, which allow the diffusion of small hydrophilic molecules across the bacterial outer membrane. The porin also acts as a receptor for a number of phages and Tol-dependent colicins, including colicins A and N, and is required for translocation of the enzymic E colicins (Bndetti *et al.*, 1989). Mutations located in the lumen of the channel modify the permeability to hydrophobic antibiotics (Benson *et al.*, 1988). The three-dimensional structure of the OmpF porin has been solved at 2.4 resolution (Cowan *et al.*, 1992). Each porin monomer consists of a β -barrel of 16 antiparallel β -strands that contains the channel. Six loops, each of 11–17 amino acids, are exposed at the surface of the outer membrane, one of which is involved in porin subunit contact. The longest loop (L3), of 34 residues, is bent into the channel and forms a constriction or selectivity gate (Cowan *et al.*, 1992). In this region a positively charged cluster, formed by basic residues that protrude from the barrel wall, face two acidic residues located on loop L3. This establishes a strong electrostatic field parallel to the plane of the outer membrane. It is interesting that one of a group of colicin-N-resistant mutants (Gly119Asp), which was isolated by *in vitro* mutagenesis of the *ompF* gene, is located in L3 and causes a change in the selectivity gate such that it is subdivided into two compartments (Jeanteur *et al.*, 1994). The Gly119Asp mutation results in a reduction in channel conductance by about one-third and a decrease in permeation rates of various sugars by factors of 10^2 – 10^{12} . Since the mutant protein appears to have the same β -barrel structure as that of the native protein as well as the same surface structure (Jeanteur *et al.*, 1994), it is tempting to speculate that colicin N (and other Tol-dependent colicins which use this porin) actually traverse the outer membrane through the OmpF porin channel. It is an intriguing question as to whether the strong transverse electrostatic field at the selectivity gate of OmpF could play a role in the unfolding of the colicin as it traverses the porin channel. The precise relationship between binding of E colicins to BtuB and their subsequent interaction with OmpF (or TolC in the case of colicin E1) is unknown.

Perhaps the most interesting protein in the Tol-dependent translocation pathway is TolA (Levengood *et al.*, 1991). It comprises three domains: the N-terminal domain of some 40 amino acids which anchors the protein in the cytoplasmic membrane, a central domain of some 260 amino acids which spans the periplasmic space, and a C-terminal domain of some 120 amino acids. Interactions between the N-terminal domain of both colicin A and E1 with the C-terminal domain of TolA have been demonstrated *in*

vitro by Western blotting of total cell proteins of *E. coli* cultures that overproduce the TolA protein, or a deletion-derivative of *tolA* (Bndetti *et al.*, 1991). After renaturation of the proteins on the filter and incubation with colicins, binding to the TolA proteins was detected by using specific antibodies. The results showed that the Tol-dependent colicins A and E1 were able to interact with TolA, whilst the TonB-dependent colicin B was not. The C-terminal region of TolA was required for the colicin binding, although the isolated N-terminal domain of colicin A, corresponding to the translocation domain, was sufficient for binding. It is tempting to speculate that the *in vitro* interaction between TolA and colicins A and E1 may be of importance in the translocation process *in vivo*. New experimental approaches to this problem have been suggested by the observation that Tol-dependent colicins contain a G-rich pentapeptide sequence close to the N-terminus which has been designated the 'TolA box' (Pilsel & Braun, 1995). The involvement of this pentapeptide sequence in Tol-dependent translocation is currently being investigated by site-directed mutagenesis.

The TonB–ExbB–ExbD complex facilitates the flow of energy from the cytoplasm to the outer membrane for the energy-dependent transport of ferric siderophore complexes and vitamin B₁₂ (Braun, 1995). It has been proposed that an energized conformation of the TonB protein opens channels in the outer membrane, formed by proteins like FepA, which acts as a receptor for the ferric siderophores, ferric-dihydrobenzoic acid (DBS), or BtuB which is a receptor for vitamin B₁₂. These receptor proteins contain a pentapeptide motif close to the N-terminus which is called the 'TonB box' (Schramm *et al.*, 1987). Mutations in the TonB box can reduce receptor activity dramatically; they can be suppressed by mutations in TonB (Braun, 1995). Such allele-specific extragenic suppression indicates physical and functional interaction between TonB and the receptors. As might be expected, group B colicins such as colicins B and D, which bind to the FepA receptor, and colicin M, which binds to the FhuA receptor, also contain a TonB box sequence close to their N-terminus. This indicates that these colicins interact directly with TonB during translocation. Mutations in the TonB box of colicin M affected its uptake and were suppressed by the Q160L and Q160K substitutions in TonB, whilst mutations in the TonB box of FhuA also reduced colicin M sensitivity. These results suggest that TonB has to interact with FhuA and colicin M for translocation to occur.

The addition of the pore-forming colicin A to *E. coli* cells results in an efflux of cytoplasmic potassium (Bndetti *et al.*, 1992b). A lag time which precedes the potassium efflux is believed to be related to the time needed for translocation of the colicin to the outer surface of the cytoplasmic membrane. Denaturing the colicin with urea before adding to the cells decreased the lag time, whilst after renaturation the lag time was similar to native colicin A. This suggests that unfolding of colicin A accelerates its translocation. The addition of trypsin, which cannot access the periplasmic space, resulted in an immediate arrest of potassium efflux, which is believed to reflect a

closing of the pores. This long-distance effect of trypsin suggests that part of the colicin A molecule may still be accessible from the external medium when the pore has formed in the cytoplasmic membrane (Bénédicti *et al.*, 1992b). It has recently been confirmed that colicin A is still in contact with its receptor and the translocation machinery upon formation of its pore in the cytoplasmic membrane (Duché *et al.*, 1995). It will be interesting to see whether a similar process occurs with the enzymic colicins, where the cytotoxic domain has to reach the cytoplasm to be active. It is of interest that a fusion protein consisting of the translocation and receptor-binding domains of colicin A with the RNase domain of colicin E3 is biologically active (Bénédicti *et al.*, 1992a). The use of mutants of colicin E9, in which mutations in the DNase domain inactivate the cytotoxic activity of the colicin (Garinot-Schneider *et al.*, 1996), will be invaluable in following the uptake of this enzymic colicin, since cells can be loaded up with large amounts of colicin without cell killing. This should improve the signal/noise ratio in immuno-electron microscopy experiments, in which antibodies against the DNase domain are used to follow the uptake of colicin E9 into sensitive *E. coli* cells. These experiments may also provide the answer to the paradoxical question of the fate of the Im9 protein when colicin E9-Im9 complex is incubated with *E. coli* cells. Given the very high stability of this complex (9.3×10^{-17} M) (Wallis *et al.*, 1995a), it is difficult to see how (and where) Im9 is released from the DNase domain to allow its cytotoxicity.

It will also be interesting to determine whether a fusion of the translocation and receptor-binding domains of a group B colicin, which is taken up by the TonB-dependent uptake system, with the DNase domain of colicin E9 produces a biologically active colicin. A hybrid toxin made from the bacteriophage f1 attachment protein and the cytotoxic RNase domain of colicin E3 was biologically active and had an altered receptor specificity (Jakes *et al.*, 1988). It is striking that the TolB protein, which is required for cell killing by colicin E3 but not for phage f1 infection, is necessary for killing by the hybrid toxin. This is perhaps suggestive of an essential role for the TolB protein in the transport of the cytotoxic domain of the enzymic E colicins. Structural studies of the Tol proteins and the determination of the affinity of binding of TolA for colicin A, E1 and the enzymic E colicins will be invaluable in furthering our understanding of the Tol-dependent translocation system.

Evolution of E colicin plasmids

The existence of four members of the DNase-type E colicins (E2, E7, E8 and E9), and of at least two members of the RNase-type E colicins (E3 and E6) have been of great value in the identification and characterization of specificity determinants in the interaction between a colicin and its cognate immunity protein. However, it raises the problem of how these families of E colicin genes evolved. There are at least three hypotheses that have been proposed to explain the evolution of colicin plas-

mids: positive selection of diversity, recombinational shuffling, and transposition.

Positive selection

The theory of positive selection of colicin diversity was proposed by Riley (1993). Mutations in the nuclease domain of an ancestral E colicin gene which give rise to a 'new' colicin are assumed to require a parallel mutation, or co-evolution, in the ancestral immunity gene to avoid killing of the host cell by the 'new' colicin, whilst maintaining immunity to the ancestral E colicin. The mutation is then rapidly fixed in the population because of the competitive advantage conferred by the ability to kill closely related cells that are competing for limited nutrients. Although the selection pressure for co-evolution of the ancestral immunity gene would be very high, it is difficult to see how there would be time for such mutations to occur since it has been assumed that host cell killing by a 'new' colicin activity would be very rapid. A possible resolution to this paradox came with the suggestion that the DNase activity of colicin E9 is not initially active inside the producing cell (Curtis & James, 1991). It is only after secretion, binding to the BtuB receptor and re-entry into the host cell that the DNase activity is growth inhibitory. This perhaps gives a window of opportunity for co-evolution of the immunity gene to occur. Our data on the affinity of non-cognate immunity proteins for the DNase domain of colicin E9 (Wallis *et al.*, 1995b) also provides support for this proposed model in that single mutations in the DNase domain of an ancestral colicin gene are unlikely to reduce radically the affinity of binding for the immunity protein. Thus, there would remain a significant level of immunity. There would however be a selection pressure from the residual killing activity of the mutant colicin to select for compensating mutations in the immunity gene in order to restore complete immunity.

Recombinational shuffling

Evidence from restriction analysis has revealed some structural similarities between the ColE2-E9 plasmids (Watson *et al.*, 1985; Chak & James, 1986). Incompatibility studies between these plasmids, measured by determining the extent of mutual exclusion, suggested two incompatibility groups. The ColE3-CA38, ColE7-K317 and ColE8-J plasmids belong to one incompatibility group, whilst the ColE5-099, ColE6-CT14 and ColE9-J plasmids belong to another (Cooper *et al.*, 1986). These plasmids require plasmid-encoded Rep proteins and host DNA polymerase I for plasmid replication. The Rep protein binds to the origin of replication and synthesizes a unique primer RNA for initiation of leading-strand DNA synthesis at the origin of replication by DNA polymerase I. The specificity of the interaction with Rep proteins is determined by the presence or absence of a single base pair in the origin. Cloned origins of replication exclude plasmids of the same incompatibility group (IncB function) due to competition for the Rep proteins. The expression of the *rep* gene is regulated negatively at a post-transcriptional step by the *incA* gene product, an antisense

RNA (RNA I), which is complementary to the 5' untranslated region of the Rep mRNA. RNA I forms a stable complex with the Rep mRNA *in vitro*. The rate of binding is affected even by single-base substitutions and results in an increase in plasmid copy-number. A detailed comparison of the nucleotide sequences of the incompatibility region of the ColE2-related plasmids reveals a mosaic of four segments which are most likely to have been created by homologous recombination and/or site-specific recombination events involving a common ancestor plasmid (Hiraga *et al.*, 1994).

Support for this hypothesis has also come from the work of Braun's group who have compared the DNA sequences of colicins A, B and D (Roos *et al.*, 1989). The receptor and translocation domains of colicins B and D are 96% identical, reflecting their common uptake route, whilst their C-terminal domains are very different, reflecting their different toxicity mechanisms. In contrast, the translocation domains of colicins A and B are completely different whilst their activity regions are highly homologous (59%) as both are pore-forming colicins. A more recent comparison of the DNA sequence of colicins E1 and E10 indicates four segments, two of which are very similar and two of which are different. The construction of hybrids between these two colicins has allowed these segments to be mapped to functional domains which support a model in which these colicins have evolved by recombination of DNA fragments that encode receptor-binding, translocation and cytotoxic activity (Pilsel & Braun, 1995).

The intriguing observation of the occurrence of dual-immunity genes in E colicin plasmids came about from the discovery of the ColE8-J and ColE9-J plasmids by Cooper & James (1984) at the University of East Anglia. Immunity testing showed that the ColE3-CA38 plasmid conferred immunity to both colicins E3 and E8, the ColE9-J plasmid conferred immunity to both colicins E9 and E5, and the ColE6-CT14 plasmid conferred immunity to both colicins E6 and E8. It has been suggested that the presence of dual-immunity genes represents a trapped evolutionary intermediate (Lau *et al.*, 1984a), presumably the result of a recombinational event between two different colicin plasmids. It is intriguing that in each case the dual-immunity genes are to an RNase (E3, E5 or E6) and a DNase type E colicin (E8 or E9).

Transposition

The discovery of a defective transposon-like structure in the ColE9-J plasmid, which appears to be associated with the insertion of a 'new' ColE9 DNase domain into the 3' end of colicin E5 as an in-frame fusion, strongly supports the role of transposition in the evolutionary relationship between the ColE5-099 and ColE9-J plasmids (Lau & Condie, 1989). From physiological studies which indicate a rapid inhibition of protein synthesis in colicin-E5-treated *E. coli* cells (Mock & Pugsley, 1982), it has been suggested that colicin E5 is an RNase. Sequence analysis however reveals no significant homology with the other RNase-type E colicins. This is the only reported example

of transposition as a mechanism for the evolution of colicin plasmids.

Future prospects

The number of fundamental biological problems that can be addressed using the colicin system is very extensive. The large-scale use of nisin in processed cheese and canned foods (Rayman & Hurst, 1984) is the first example of the use of a low-molecular-mass polypeptide bacteriocin, produced by *Lactococcus lactis*, as a food preservative. Interest in 'natural products' rather than man-made chemicals should lead to further opportunities for the use of bacteriocins in areas such as the control of plant diseases of bacterial origin, and perhaps even in medical applications where bacterial infections are difficult to control and where the potential immunological complications of using polypeptides are not insurmountable. Examples of this kind include the control of dental caries caused by *Streptococcus mutans* (Loyola-Rodriguez *et al.*, 1992), and even in the treatment of cystic fibrosis where the growth of *Pseudomonas aeruginosa* in the lungs causes severe breathing difficulties. Bacteriocin production may well be involved in the invasion of bacteria in the cystic fibrosis lung (M. Riley, personal communication). The use of protein engineering techniques in order to improve the BRP system to facilitate the extracellular release and purification of a number of recombinant-DNA-encoded proteins is another interesting development (van der Wal *et al.*, 1995).

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